tion by degradation products. The preparation was therefore repeated, using Barnett's method [1921] in which sulphuryl chloride is employed as a catalyst (thereby reducing degradation to a minimum) and was carried out according to the modification used by Irvine & Hirst [1922] for the preparation of cellulose tri-acetate. 2 g. sclerotiose were soaked in 10 ml. glacial acetic acid, through which a stream of dry sulphuryl chloride had been passed for 30 sec. After setting aside for ½ hr., 122 ml. of acetic anhydride were added and SO_2 was then passed through the liquid for 1 min. At the end of 1 hr., during which the pasty mixture was vigorously stirred, the temperature was raised to 65° and maintained there until the polysaccharide had all dissolved to give a clear, colourless, viscous solution. After cooling to 30°, an equal volume of $CHCl_3$ was added, followed by a large excess of water. The mixture was continuously stirred and the $CHCl_3$ evaporated. During this process the acetate separated in the form of fine white granular particles, which were obtained as an aggregate of microcrystals on slow evaporation of the $CHCl_3$ solution. M.P. 165–168° (decomp.). (Found: C, 49·6; H, 5·8; O, by difference, 44·6%. The tri-acetate $C_6H_7O_5(OC.CH_3)_3$ requires C, 50·0; H, 5·5; O, 44·5%.)

There was no appreciable loss of material by this method. The filtrate had no action on Fehling's solution, and on evaporation to dryness gave a negligible residue; it was optically inactive. The specific rotation of sclerotiose tri-acetate, in $CHCl_3$ solution, was $[\alpha]_D = +205·7$, while the acetic acid content was 62·1% (calc. 62·5%).

Sclerotiose benzoate

Benzoylation was carried out by a modification of the Schotten-Baumann reaction. The final product had a benzoic acid equivalent of 65·6%. It was therefore probably the tri-benzoate, but contained some di-benzoate.

SUMMARY

1. Investigation of the rapid glucose consumption of *P. sclerotiorum*, on Czapek-Dox and Raulin-Thom media, led to the isolation of a polyglucose, somewhat similar to other polysaccharide metabolic products of the lower fungi.

2. The influence of the culture medium and its *p*H were amply demonstrated by the varying yield of sclerotiose. As expected the neutral medium gave the greater proportion of polyglucose, the more acid substrates having a hydrolysing effect [Reilly & Curtin, 1943]. Of the derivatives prepared, the primary or tri-derivative was always present in preponderating amount.

The authors wish to thank Prof. J. Reilly for his continued help with this work.

Part of the expense of this investigation was met by a grant from the Industrial Research Council, Eire, to one of us (D. R.).

REFERENCES

Baker, J. L. & Houlton, H. F. E. [1920]. *Biochem. J.* **14**, 754.
Barnett, W. L. [1921]. *J. Soc. chem. Ind., Lond.*, **40**, 8T.
Birkinshaw, J. H., Charles, J. H. V. & Raistrick, M. [1931]. *Philos. Trans.* A, **220**, 355.
Curtin, T. P. & Reilly, J. [1940]. *Biochem. J.* **34**, 1419.
Dox, A. W. & Niedig, R. E. [1914]. *J. biol. Chem.* **18**, 167.
Irvine, J. C. & Hirst, E. L. [1922]. *J. chem. Soc.* **122**, 1587.
Judd, M. [1920]. *Biochem. J.* **14**, 754.
Neuberg, C. [1899]. *Ber. dtsch. chem. Ges.* **32**, 3384.
Pictet, A. & Reilly, J. [1921]. *Helv. chim. Acta*, **4**, 613.
Reilly, D. & Curtin, T. P. [1943]. *Biochem. J.* **37**, 36.
Reilly, J., Hayes, M. & Drumm, P. J. [1931]. *Proc. R. Irish Acad.* **8**, 102.
Ward, G. E., Lockwood, L. B., May, O. E. & Herrick, H. T. [1935]. *Industr. Engng Chem.* **27**, 318.
Willstätter, R. & Schudel, G. [1918]. *Ber. dtsch. chem. Ges.* **51**, 780.

The Excretion of a Metabolic Product of Salicylic Acid

By CECILIA LUTWAK-MANN (Fellow of Newnham College, Cambridge),
From the Biochemical Laboratory, Cambridge

(*Received 23 December 1942*)

The experiments described below are a continuation of the work by Lutwak-Mann [1942].

METHODS

Alkali test in urine. It was shown previously that shortly after the injection of salicylate there appears in rat urine a substance, which on addition of fairly strong NaOH, and in the presence of air, causes the urine to turn brown to black. The following modifications of this test have now been worked out. When only very small amounts of urine are available 1–2 drops of it are added to about 4–6 ml. NaOH (*M*/50 or stronger). After a 30–60 sec. lag a pink colour develops which persists for varying lengths of time (2–5 min.), after which the solution turns yellow to brown. Excess of either urine or alkali prevents the appearance of the pink phase and only the final brown colour is seen. A still more reliable result is obtained by acidifying a few ml. of urine with 10% HCl and shaking it with 3 vol. ether. The separated ether extract is then shaken with a few ml. dilute NaOH. The aqueous solution turns at once a bright pink colour which persists for a short while and then the

solution becomes brown. A very small trace of the alkali-sensitive substance excreted in urine after the administration of salicylates can be detected in this manner.

Isolation from urine of the substance responsible for the alkali test. Six large male rats (300 g.) were injected every second day with a mixture of 0·3 mg. Na-salicylate plus 0·3 mg. Na-aspirin/g. wt. until the animals had received a total of 3·2 g. salicylates. The urine collected during this period (450 ml.) was acidified with HCl and extracted with ether repeatedly till a sample of the extracted urine failed to give a positive alkali test. The ether extract was washed with dilute $NaHCO_3$ till the washings became colourless. The aqueous solution was divided into two, half of it was acidified and again extracted with ether (fraction A). The rest was adjusted to *p*H 6·8 and Pb acetate was added, avoiding excess. The Pb precipitate was discarded as it was found that the supernatant alone gave the alkali test. The Pb-containing fluid was left at room temperature for 2 days when it was found that the alkali-sensitive substance could no longer be extracted by ether though it could be extracted with ethyl acetate (fraction B).

Fraction A contained a great deal of salicylic acid as well as the substance responsible for the NaOH test. It was noticed, however, at this stage, that when dilute $FeCl_3$ was added to a sample a distinct blue tinge developed though it was somewhat obscured by the purple colour characteristic of salicylic acid. When salicylic acid was removed by extraction with chloroform and benzene, a whitish powder (0·15 g.) was obtained from this fraction, which showed the following properties. It was acid in aqueous solution, and turned intensely blue with dilute $FeCl_3$; on addition of NaOH it became pink and then slowly went brown, thus resembling the reactive substance in the urine. It reduced Fehling's solution on heating, ammoniacal $AgNO_3$ in the cold, and phosphomolybdic acid on prolonged standing. It was fairly soluble in hot water, very soluble in ethanol and ether, but insoluble in chloroform, benzene and CS_2. On recrystallization from water the M.P. 198° was obtained. The properties of the substance isolated which is responsible for the behaviour of salicylate urine towards NaOH, resemble those of 2:5-dihydroxybenzoic or gentisic acid. Using a specimen of gentisic acid prepared by the method of Graebe & Martz [1905] which showed a M.P. 199°, a mixed M.P. of 198° was obtained; owing to the author's having taken up war work it was not possible to carry out the full analysis of the product obtained from urine. The assumption, however, that this substance is gentisic acid is further strengthened both by the older findings of Angelico [1921], Baldoni [1908], and Neuberg [1911], as well as by the recent work of Kapp & Coburn [1942], who describe the isolation and identification of gentisic acid from human urine as one of the urinary metabolites of Na-salicylate.

From the Pb-containing ethyl-acetate fraction B, after washing with water, a solution was obtained which gave a strong test with NaOH and reduced Fehling's reagent, but which gave only a pinkish brown colour with $FeCl_3$. When this fraction was concentrated on a water-bath and cooled in ice it turned into a transparent gel, which subsequently solidified into a white crystalline mass. After recrystallization from dilute ethanol 0·48 g. of white crystalline needles was obtained. The behaviour of this substance towards NaOH and Fehling's reagent, as well as its extremely poor solubility in cold water, are analogous to those of the Pb salt of gentisic acid [Senhofer & Sarlay, 1882]. For reasons mentioned above, however, its analysis could not be completed.

An attempt was made to show the *in vitro* formation of gentisic acid from salicylate. The following samples were set up and incubated for 2 hr. at 38° in $O_2 + CO_2$ (95 + 5%): (*a*) 25 g. liver slices (rat), (*b*) 18 g. liver pulp, (*c*) 15 g. minced skeletal muscle, all of them containing *M*/50 salicylate, *M*/30 phosphate buffer of *p*H 7·3, and 0·3% bicarbonate. From the acid-ether extracts of the muscle mince and the liver pulp a chloroform-insoluble fraction was obtained which, dissolved in ethanol, gave a distinctly blue colour with dilute $FeCl_3$, and slightly darkened on addition of NaOH. No such definite result could be obtained in the extracts from the liver slices where substances may have been present which interfered with these tests.

The substance isolated from urine, presumably gentisic acid, was examined and found to be stable in the presence of the enzymes of rat liver, kidney and muscle, as well as of baker's yeast. It was not oxidized by cytochrome or catechol oxidase. Peroxidase slowly but steadily oxidized it in presence of H_2O_2.

Substances which give the alkali test in urine. The following substances related to salicylic acid were administered to rats: acetylsalicylate (Na-aspirin), methylsalicylate (oil of wintergreen), phenylsalicylate (salol), Na-sulphosalicylate. Their effect on the urine excretion was examined as described in previous work. All of them caused an increase in the reducing power of urine (as measured by titration against 2:6-dichlorophenol indophenol) presumably owing to an increased excretion of ascorbic acid, but the NaOH test was positive only after aspirin and methylsalicylate injections. The alkali test in urine after aspirin injection was always stronger than after a similar dose of salicylate. After the injection of methylsalicylate early urine samples (0–7 hr.) did not exhibit a positive alkali test as they did after salicylate or aspirin treatment; the later samples, however (7–24 hr.), showed a strongly positive test.

Substances which prevent the formation of gentisic acid from salicylates. Previous work has shown that large doses of ascorbic acid are without effect on the excretion in urine of the substance formed from salicylic acid which is responsible for the alkali test, though such doses completely abolished the excretion of homogentisic acid in tyrosine alkaptonuria. In this work the effect was studied of poisons such as white phosphorus (P), carbon tetrachloride (CCl_4) and phlorrhizin upon the excretion of gentisic acid after salicylate treatment.

(*a*) *White phosphorus.* A group of 10 rats received 0·1 mg. white P in olive oil every 2 days for a fortnight. At the end of this period autopsy showed fatty infiltration of the liver; the titratable reducing power of the urine increased considerably, and all the rats excreted large amounts of urobilin. Some of them were then injected with salicylate, others with Na-aspirin (0·5 mg./g.). Urine analysis showed the following facts: that no NaOH test was

given, either soon after the injection or in later urine samples; that the titratable reducing power was still further increased, but that there was no rise in the *p*H of the early samples and no albuminuria, though these were both previously found to be characteristic features of urine from salicylate-treated normal rats.

The effect of acute P poisoning was also studied. The rats received only 2 doses of P (1·5 mg.) in the course of 3 days, after which they were given salicylate and aspirin by injection. Although there was no urobilinuria, and as yet no marked changes in the liver on autopsy, these rats failed entirely to excrete gentisic acid, as judged by negative alkali tests in their urine.

Both the chronically and the acutely poisoned rats were kept for another 10 days on a normal diet without white P. Urobilinuria continued unabated, but the majority of the animals survived, and those rats which had been treated with salicylates seemed to recuperate more quickly than the others. At the end of this period the injections of salicylate and aspirin were repeated. This time all of them excreted urine which gave a strongly positive test with NaOH, i.e. recovery had occurred.

(*b*) *Carbon tetrachloride.* Injections of CCl_4 (0·4 ml./200 g.) were given every other day to rats. After 14 days of this treatment, which was remarkably well tolerated, they received injections of salicylate and aspirin (0·5 mg./g.). Of eleven CCl_4-poisoned rats, eight failed to excrete the substance responsible for the alkali test, but the urine of the remaining three animals showed a faintly positive test with NaOH. The titratable reducing power of the urine, which fell conspicuously during the CCl_4 treatment, rose in the usual manner in response to salicylates. When the CCl_4 injections were discontinued for a few days and the administration of salicylate and aspirin repeated, all eleven rats excreted urine which gave a strong NaOH test.

Experiments were made with the P- and CCl_4-poisoned rats in which anthranilate (*o*-amino-benzoate) was used instead of salicylate. It had been shown previously that the administration of anthranilate to rats caused the excretion in urine of a substance which in presence of alkali (NH_4OH) turned purple, and that in addition the urine contained considerable quantities of a glucuronide. When the poisoned rats were given anthranilate they continued to produce the glucuronide, but the test with NH_4OH was completely negative.

(*c*) *Phlorrhizin.* This was given by injection (30 mg./200 g.). Three hours later when glucosuria was fully developed some of the rats were given salicylate by injection, others aspirin (0·5 mg./g.). The NaOH test was greatly increased in all urines, and that from the aspirin-treated rats turned almost black on addition of NaOH. The titratable reducing power, however, was rather lower than in non-phlorrhizinized animals.

The alkali test in human urine. Urine was collected and examined after an oral dose of aspirin (1·2 g.). The acid-ether extract from the early urine samples (0–7 hr.) showed no alkali test, but the 7–15 hr. samples, which also showed a slight increase in the titratable reducing power, gave a definite test. Later samples (15–24 hr.) showed only a mere trace of the test.

SUMMARY

1. A substance previously reported to be present in rat urine after the administration of salicylate, and which causes the urine to turn dark on addition of NaOH in air, has been isolated. Its properties are found to agree with those of 2:5-dihydroxybenzoic acid (gentisic acid).

2. Tests are described by means of which traces of this substance can be demonstrated in urine.

3. Positive tests of this kind were obtained after the administration of salicylate, aspirin and methylsalicylate, but not after salol or sulphosalicylate. Human urine gave a positive test after a comparatively small dose of aspirin.

4. The substance isolated from urine of salicylate-treated rats, which is thought to be gentisic acid, is stable in the presence of rat tissue, and of yeast enzymes. It was oxidized by peroxidase, but not by cytochrome or by catechol oxidase.

5. Rats poisoned with white phosphorus or carbon tetrachloride are unable to excrete gentisic acid after administration of salicylates. However, the effects of the poisoning are not permanent, since when the poisons are discontinued for a period, recovery sets in and administration of salicylates is then again followed by the excretion of this particular product of salicylate metabolism. On the other hand, phlorrhizin does not prevent, but rather increases, the excretion of the alkali-sensitive substance in the urine after administration of salicylates.

The work on the effect of the anti-rheumatic drugs was originally undertaken on behalf of the Empire Rheumatism Council.

REFERENCES

Angelico, F. [1921]. *Arch. Farmacol. sper.* **31**, 8.
Baldoni, A. [1908]. *Arch. Farmacol. sper.* **8**, 174, 193.
Graebe, C. & Martz, E. [1905]. *Liebigs Ann.* **340**, 213.
Kapp, E. M. & Coburn, A. F. [1942]. *J. biol. Chem.* **145**, 549.
Lutwak-Mann, C. [1942]. *Biochem. J.* **36**, 706.
Neuberg, C. [1911]. *Berl. klin. Wschr.* **48**, 799.
Senhofer, C. & Sarlay, F. (1882). *Mh. Chem.* **2**, 442.